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A neuronal cell-based botulinum neurotoxin assay for highly sensitive and specific detection of neutralizing serum antibodies

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Abstract ***Clostridium botulinum*** **neurotoxin (BoNT) serotypes A and B are widely used as pharmaceuticals to treat various neurological disorders and in cosmetic applications. The major adverse effect of these treatments has been resistance to treatment after multiple injections. Currently, patients receiving BoNT therapies and patients enrolled in clinical trials for new applications and/or new formulations of BoNTs are not routinely monitored for the formation of neutralizing antibodies, since no assay other than the mouse protection procedure is commercially available that reliably tests for the presence of such antibodies. This report presents a highly sensitive and specific neuronal cell-based assay that provides sensitive and specific detection of neutralizing antibodies to BoNT/A.**

Keywords: Botulinum neurotoxin; BoNT; Serum antibody; Cell-based assay; Spinal cord cell

1. Introduction

Botulinum neurotoxin (BoNT) produced by neurotoxigenic clostridia are the most potent naturally occurring toxins known [1]. Based on their antigenic specificity, BoNTs are distinguished into seven serotypes (A–G) [2], with BoNT/A, B and E accounting for nearly all recorded cases of human botulism [1,3]. BoNTs are zinc-containing metalloproteases of ca. 150 kDa consisting of a heavy chain (~100 kDa), and a light chain (~50 kDa) linked by a disulfide bond. The C-terminal domain of the heavy chain functions in receptor binding on the neuronal cell surface, inducing endocytotic internalization of the toxin. Once inside the endosome, protonation causes membrane insertion and chaperone/channel formation of the heavy chain coupled to light chain unfolding and entry into the channel. This is followed by light chain conduction through the heavy chain channel and subsequent release of the light chain by disulfide bond reduction and light chain refolding in the cytoplasm of the cell [4,5]. Light chains of BoNTs are zinc endopeptidases that target core proteins including SNAP-25, VAMP/synaptobrevin, and syntaxin 1 involved in trafficking and release of neurotransmitters [6–11].

The high potency of BoNT, its high specificity for motor neurons, and the longevity of its action (up to several months) have facilitated the use of BoNT/A and/B as extremely valuable drugs for treatment of a myriad of neurological diseases, as well as for cosmetic treatments, with BoNT/A being the most prominent serotype currently used [12]. Despite the effective use of BoNTs in clinical applications, the major adverse effect has been the formation of antibodies which render patients refractory to treatment and tachyphylaxis [13–15]. For example, 5–10% of patients with cervical, segmental or multifocal dystonia receiving repeated BoNT/A treatments were estimated to develop resistance to treatments due to the presence of circulating neutralizing serum antibodies [14,15]. Resistance to BoNT treatment can be confirmed in a clinical setting by test injecting BoNT into the patient's frontalis muscle, extensor digitorum brevis (EDB) or sternomastoid muscle [16–20], and measuring compound muscle action potentials. However, patients are not routinely monitored for antibody formation during their treatment regime, since a sensitive assay that measures neutralizing antibodies in human sera is not commercially available [21]. Such monitoring is highly desirable in clinical trials of BoNTs as well as for currently approved therapies.

Several laboratory assays for the detection of BoNTs and BoNT specific antibodies have been developed. The in vivo mouse bioassay currently is the standard method to detect BoNT activity, and the only assay approved by the FDA [22,23]. In this assay, mice are injected intraperitoneally or intravenously with toxin or toxin/antibody mixtures and observed for signs of toxicity and death. While this assay is well established and quantitative, it is relatively insensitive and has well-known drawbacks including the need for a large number of animals and associated required facilities and expenses, the requirement for 2–4 days for results, non-specific deaths, and the need to use mice.

Alternative in vitro assays include the mouse diaphragm assay or MDA [22], enzyme-linked immunosorbent assays (ELISAs) and variations, immunoprecipitation assay (IPA), chemiluminescent slot blot immunoassay, electro chemiluminscence, radioimmunoassay, lateral flow immunoassays, endopeptidase assays and others [25]. All of these assays can be used to quantitate BoNT's in vitro and in foods and clinical samples [22,26–28]. However, many have the drawback of high background, and most measure only one biological property of BoNT activity (binding of the toxin to antibody, or proteolytic activity in the endopeptidase assays). In order to reliably measure BoNT holotoxin activity and detect neutralizing serum

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antibodies, an assay should simulate all aspects of intoxication (i.e., binding of the heavy chain binding domain to the cell surface receptor, endocytosis, channel formation, conductance of the light chain into the cell's cytosol and disulfide bond cleavage, refolding of the light chain, and proteolytic cleavage of the target protein within the cell by the light chain).

A more complete approach for the screening of neutralizing antibodies as well as potency determination of the holotoxin is the use of cell-based BoNT assays. Several cell-based assays have been developed, including continuous cell lines such as neuro-2a, PC12, or SK-N-SH cells [8,29–31], as well as primary neurons derived from chicken, mouse or rat spinal cord cells [32–39]. Successful detection of BoNT can be achieved by Western blot assay of the cleaved target protein [30,34–36,38], by specific FRET sensors [29], or by neuronal activity testing [31,33,37,39]. However, continuous cell lines exhibit very low BoNT sensitivities and therefore cannot be used for detection of serum antibodies. Most primary neuronal cell assays using pure BoNT/A preparations have been reported to exhibit sensitivities of approximately 50–100 pM of BoNT/A (~250–750 mouse LD_{50} U), which is not sufficient for detection of most human serum antibodies. One primary cell assay has been adapted to detect as little as 3 pM BoNT/A and protection by up to 0.001 IU/ml of Equine International sera by measuring [^{3}H] glycine release from primary rat spinal cord cells [33]. However, enhanced practicality and even higher sensitivity and specificity are desired for clinical and research applications.

The assay presented here detects as little as 33 fM BoNT/A and E (~0.1 mouse LD_{50} U), and requires each step in the intoxication process as it measures changes in intracellular substrate cleavage and therefore all preceding steps of the intoxication process must have already occurred. Using human serum samples, the usefulness of the assay in detection of neutralizing serum antibodies is presented and compared to the mouse lethality assay.

2. Materials and methods

2.1. Botulinum neurotoxin and mouse bioassay

Pure botulinum neurotoxin (BoNT) A, B, and E (150 kDa) were prepared from *Clostridium botulinum* strains Hall A hyper, Okra B, and Beluga E as previously described [40,41]. The toxins were dissolved in phosphate buffered saline, pH 7.4 and 40% glycerol, and stored at −20 °C until use. Activity of the BoNT/A,/B, and/E preparations were determined by the mouse bioassay [22,23], and specific toxicity was about 10^8 mouse LD_{50} U/mg.

To estimate the titer of serum samples by mouse lethality assay [33], 75 μl of serum were pre-incubated with 125 pg (and indicated dilutions) of BoNT/A in a total volume of 0.3 ml for 1 h. Each dilution was injected intraperintoneally into at least two mice, and the mice were observed until death for up to 4 days.

2.2. Human sera samples

Human sera samples from patients repeatedly treated with BOTOX® who have demonstrated complete secondary resistance (non-response), partial reduction in response, and continued effective responses were obtained from clinics after patient consent. An additional serum sample from a subject who received three doses of the pentavalent vaccine 16 years ago was analyzed (#11). All sera were stored at −20 °C until use and subsequently at 4 °C. Table 1 summarizes the relevant medical histories of the patients. The sera were labeled 1–15, and were used in a blinded manner.

2.3. Primary rat spinal cord (RSC) cells

The preparation of spinal cord cells was based on a previously described protocol with modifications as described [42]. A pregnant Sprague Dawley rat (Harlan Sprague–Dawley) at the gestational stage of

Table 1
Patient histories

Patient	BoNT/A exposure and indication	Response history	Results of remote point testing (20 U)	Mouse bioassay
1	Over 12 injections for the Treatment of Glabellar Wrinkles (Botox)	Excellent result for 7 years followed by complete loss of effect	Frown lines not responsive	Negative
2	Control	Control	Control	Control
3	Essential blpeharsoapsm – injections between 1989 and 1992 (50–100 U Botox)	Secondary none response in 1993. Botox nor Dysport	No effect on frontalis muscle (positive for resistance)	Positive
4	Cervical dystonia – 16 injections between 1992 and 1996 (200–300 U Botox)	Substantial Secondary reduced response	No effect (positive for resistance)	Positive
5	Control	Control	Control	Control
6	Control	Control	Control	Control
7	Cervical dystonia 200–250 U of Botox over 3 month intervals 1995–1998	Complete loss of response after 3–4 years to type A neurotoxin, now treated with type B	No effect (positive for resistance)	Positive
8	Cervical dystonia, receive eight injections since 1991	Loss of response after 4 years (secondary non-responder)	ND	Positive
9	Cervical dystonia, treated with 200–300 U over 14 years	Retains an excellent sponse	Sternomastoid shrinks with injections	Negative
10	Control	Control	Control	Control
11	Vaccinated subject (three doses of pentavalent vaccine in 1991–1992)	No response in remote point test	No effect on frontalis muscle in 2004	–
12	Control	Control	Control	Control
13	Cervical dystonia, eight injections between 1991 and 1995	Response 50% reduced since initiated (secondary reduced responder)	No effect or substantially reduced effect on frontalis muscle	ND
14	Control	Control	Control	Control
15	Essential blepharospasm	Continues to have benefit from repeated injections	Frontalis muscle weak after injection	Negative

E15 was euthanized by exposure to CO_2, and the uterus containing the pups was removed and placed into a dish containing dissection medium (Hanks balanced salt solution, 10 mM HEPES, 20 mM glucose (Invitrogen)). Working in dissection medium, the pups were removed from the uterus, immediately decapitated, the spinal cords were dissected out of the pups, and the membranes and ganglia surrounding the spinal cords were removed. The spinal cords were transferred to 4.4 ml of fresh dissection medium, minced, and transferred to a sterile 15 ml tube. Six-hundred millilitre of TrypLE express (Invitrogen) was added, and trypsinization was allowed to take place for 20 min at 37 °C in a 5% CO_2 atmosphere. The trypsin solution was removed and the spinal cords were washed once by addition of 15 ml of dissection medium. After the tissue settled to the bottom of the tube, the dissection medium was removed and 1 ml of culture medium pre-warmed to 37 °C (Neurobasal medium supplemented with B27, glutamax, and penicillin/streptomycin (all from Invitrogen)) was added. The cells were dissociated by pipetting up-and-down 10–12 times, and live cells were counted by trypan exclusion assay. Cells (400 000) were plated into each well of collagen coated 24-well dishes (BD BioSciences). The cells were allowed to differentiate in culture at 37 °C in a humidified 5% CO_2 atmosphere for at least 18 days with bi-weekly changes of culture medium before they were used in the toxin assay. For microscopy, cells were plated onto collagen coated cover slips (BD BioSciences).

2.4. Cell-based BoNT assay

After the differentiation period, various quantities of BoNT were added to culture medium in a total volume of 300 µl per well, followed by incubation at 37 °C in a humidified 5% CO_2 atmosphere for 48 h. To test for serum antibody neutralization, 75 µl of serum (or dilutions of serum in culture medium where indicated) were pre-incubated with 125 pg of BoNT/A (or as indicated) in a total volume of 300 µl per sample at 37 °C, 5% CO_2 for 1 h prior to exposure of the cells. After 48 h, the cells were lysed in 150 µl of 1 × LDS lysis buffer (Invitrogen). The samples were analyzed by SDS–PAGE gel electrophoresis on 12% NuPAGE Novex Bis–Tris gels in NuPAGE MOPS running buffer (Invitrogen), followed by transfer onto an Immobilon PVDF membrane (Millipore). Full length and cleaved SNAP25 or VAMP were detected with a monoclonal antibody to SNAP25 or VAMP1 (Synaptic Systems) and the chemiluminescent Western Breeze kit (Invitrogen) and exposure to X-ray film (Kodak). To quantitate full length and cleaved SNAP25 bands, the films were scanned on a Gel DOC system (BioRAD), and the bands were quantified by densitometry using Quantity One software (BioRAD).

3. Results

3.1. BoNT sensitivity of RSC cells

In order to determine when the spinal cord cells were differentiated and ready and sensitive to BoNT, the cells were periodically examined by light microscopy at 5–25 days after plating (Fig. 1). After 15 days, the appearance of cells did not change significantly, and no significant change in BoNT/A sensitivity was observed, even after 8 months (data not shown).

To determine BoNT sensitivity, RSC cells were exposed to serial dilutions of BoNT/A, B, and E, incubated for 48 h, and cleavage of the target protein (SNAP25 for BoNT/A and E, and VAMP/synaptobrevin for BoNT/B) was examined by Western blot. A weak band corresponding to the SNAP25 cleavage product was observed with as little as 1.5 pg of BoNT/A or E (33 fM or approximately 0.1 mouse LD_{50} U), and the relationship of cleaved versus full length SNAP25 with increasing BoNT concentration was linear in the range of 6–125 pg BoNT/A and 12.5–125 pg BoNT/E. At 125 pg BoNT/A (2.8 pM; ca. 10 mouse LD_{50} units), ca. 70–75% of the SNAP25 was present in the cleaved form (Fig. 2a and b).

BoNT/B intoxication of the cells was determined by using an antibody that recognizes only the full length VAMP/synaptobrevin, but not the BoNT/B cleavage product (Synaptic Systems) (Fig. 2c), and syntaxin as a loading control. A decrease in the VAMP/synaptobrevin band was apparent at 250 pg BoNT/B (5.5 pM), and the band had almost completely disappeared at 1 ng BoNT/B (22 pM).

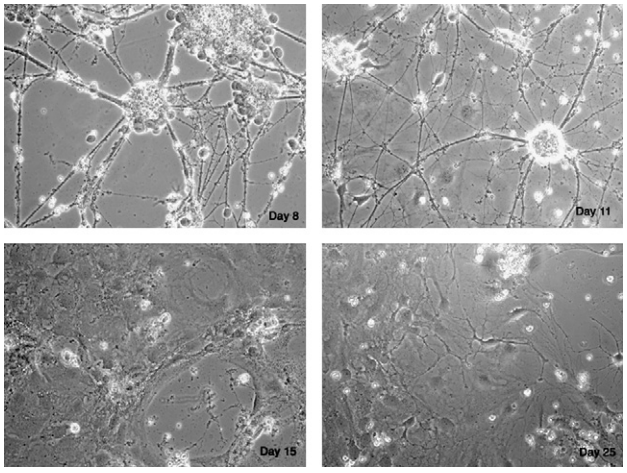

Fig. 1. Differentiation of primary rat spinal cord cells over time. Cells were plated onto collagen coated cover slips and observed by light microscopy for up to 25 days after plating.

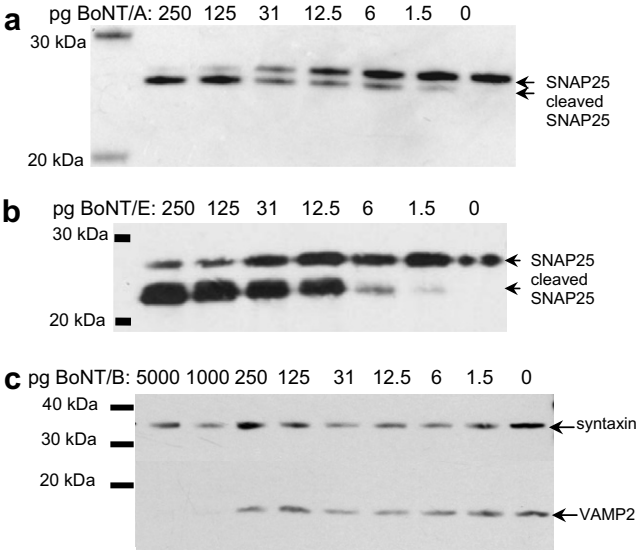

Fig. 2. Western blots showing BoNT sensitivity of RSC cells. RSC cells were exposed to serial dilutions of BoNT/A (a), E (b), and B (c), and cell lysates were examined by Western blot for SNAP25 (for A and E) or VAMP (for B) cleavage.

3.2. Detection of neutralization of BoNT/A activity by human sera in the RSC assay

To determine whether the assay correlated with clinical data of BoNT/A resistance of patients, 15 human serum samples were tested in a blinded manner. In this assay, 75 µl of the serum samples were mixed with 125 pg of BoNT/A in a total volume of 0.3 ml, and the mixture was pre-incubated at 37 °C for 1 h before exposure to the RSC cells.

In 5 of the 15 samples (#1, #4, #7, #11, and #13), no SNAP25 cleavage product was detected on the Western blots,

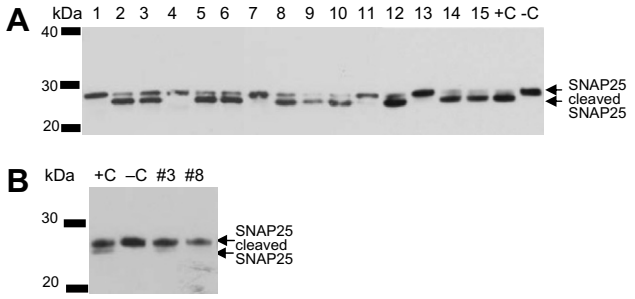

Fig. 3. Protection against SNAP25 cleavage in RSC cells by human sera. RSC cells were exposed to A: a mixture of 125 pg BoNT/A and 25% human serum of 15 different patients, and B: a mixture of 12.5 pg BoNT/A and 25% of human serum #3 and #8, and the cell lysates were assayed for SNAP25 cleavage by Western blot. The +C represents toxin only controls and the −C represents cells not treated with toxin or serum.

indicating complete protection against BoNT/A induced SNAP25 cleavage (Fig. 3). In two samples (#3 and #8), only a small reduction in the ratio of cleaved versus full length SNAP25 was observed, and this was confirmed by repeating the assay in triplicates (not shown). Additionally, in a repeat RSC assay using only 12.5 pg of BoNT/A and 75 μl of serum #3 and #8, nearly all full length SNAP25 was detected on the Western blot, indicating protection against SNAP25 cleavage. No protection was observed using these two serum samples in an independently performed mouse lethality assay. In all other samples, no significant difference to the control (no serum) was observed. These data were in excellent agreement with clinical findings of resistance to BOTOX® treatment and remote point testing (see Table 1).

3.3. Determination of sensitivity of the RSC assay for antibody detection

In order to determine the detection limit of this assay for neutralizing serum antibodies, serial dilutions of serum #4 were examined in the RSC assay in a blinded manner. Serum #4 was chosen because patient history indicated that it had the highest titer and therefore allowed for analysis of the greatest dilution range. In parallel, the serum was titered by mouse lethality assay using the same amount of BoNT/A (125 pg or ca. 10 LD_{50} U in 0.3 ml). The MLA was able to detect protection against BoNT/A induced death with serum dilutions of up to 1:120 (50% of mice died). Higher dilutions resulted in death of all mice tested (data not shown). One International Unit (IU) can neutralize 10^4 mouse LD_{50} U of BoNT/A, therefore 0.3 ml of this serum neutralizes 1200 U, and the titer of the serum was estimated to be 0.4 IU/ml.

Using the RSC assay, significant protection against cleavage of SNAP25 was observed with serum dilutions of up to 1:1600. The relationship of serum amount and SNAP25 cleavage was linear within the range of serum dilutions of 1:200 and 1:3200, with a Pearson coefficient of −0.99 (Fig. 4). This indicates that this assay can be used to reliably and quantitatively determine the neutralizing BoNT/A antibody titers of human sera.

Based on these data, the titers of three serum samples were determined using this assay. All three serum samples were derived from patient #11 at different times after a test injection with 20 U of Botox®, to which the patient was non-responsive. The titer was similar in all three serum samples and was estimated at 0.006–0.0075 IU/ml by correlation to data from dilutions of serum #4. An independently performed mouse lethality assay confirmed the titers of these samples (data not shown).

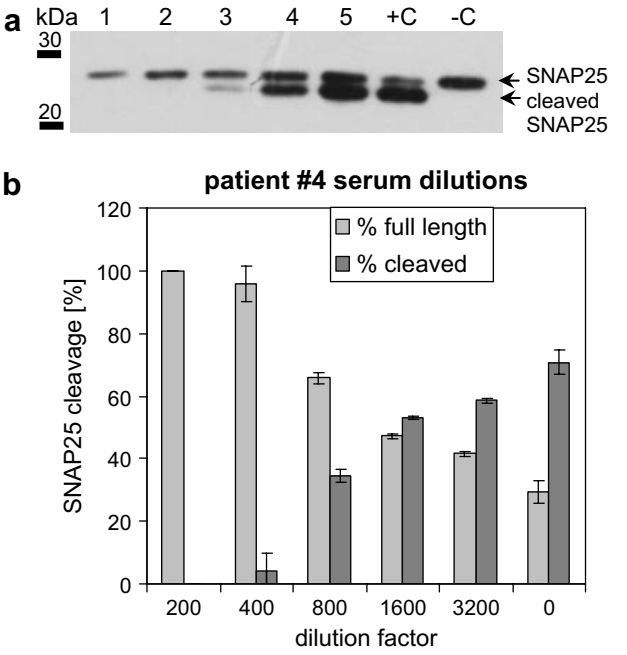

Fig. 4. Sensitivity of RSC cells for antibody detection. RSC cells were exposed to a mixture of 125 pg BoNT/A and a (1) 1:200, (2) 1:400, (3) 1:800, (4) 1:1600, (5) 1:3200 dilution of human serum #4. A Western blot of SNAP25 cleavage (a) and a quantitative representation of densitometric analysis of three Western blots (b) are shown. The +C represents 125 pg BoNT/A without serum, and the −C contains no toxin and no serum.

4. Discussion

The cell-based BoNT assay presented here is specific and highly sensitive for BoNT/A, B, and E potency determination (about 0.1 mouse LD_{50} U of BoNT/A and E), and is the most sensitive assay reported in quantitative detection of neutralizing human serum antibodies to BoNTs. Compared to the mouse bioassay/lethality assay, this assay has the following advantages: (1) no need for large numbers of animals, (2) higher specificity due to the use of BoNT substrate cleavage as endpoint, (3) higher sensitivity in detection of neutralizing antibodies, (4) excellent reproducibility with low standard deviations, and (5) increased safety for laboratory workers as toxin does not need to be handled in syringes. While this assay still requires the use of relatively few animals in order to prepare the primary spinal cord cells, one rat on average yields enough cells for 72 assays, and a small number of replicas per sample (3–5) are sufficient to yield reliable results. Currently, the time required to complete the assay is approximately equal to the mouse bioassay (3–4 days). Future refinements of this assay will determine optimal parameters such as toxin concentration and preparation, number of replicas per sample, incubation time, buffer composition, serum sampling and storage.

Other assays utilizing primary spinal cord cells have been reported previously; however, they appear to either lack the sensitivity required to be a valid replacement of the mouse bioassay, do not utilize a BoNT specific endpoint, or are impractical for routine testing [32,33,35–38]. It has been reported that most sera of patient's refractory to BoNT treatments have titers in excess of 0.001 IU/ ml [24]; however, lower concentrations still may have an impact on clinical response. Only one assay previously reported is sensitive enough to detect 0.001 IU/ml [31]. However, it utilizes a non-BoNT specific endpoint (neurotransmitter release), and requires the use of radioisotopes. The assay described here utilizes a highly specific endpoint (BoNT specific substrate cleavage), can reliably quantify serum titers down to 0.0003 IU/ ml, and can detect even lower levels by decreasing the quantity of toxin employed. In fact, the sera of two patients examined contained titers too low to be detected by the mouse lethality assay or by the RSC assay using 125 pg of BoNT/A, however, the use of only 12.5 pg of BoNT/A resulted in the detection of neutralizing antibodies (Fig. 3). These patients were resistant to BOTOX® treatments, although they had not received any treatments in over 10 years. This emphasizes the clinical importance of detecting very low antibody titers (below 0.001 IU/ml). The ability to detect such low levels of antibodies may also prove extremely useful in monitoring of patients for developing BOTOX® resistance.

Future studies are underway analyzing a larger number of serum samples to further validate and refine this assay. Even though this assay currently has lower sensitivity for BoNT/B (about 20 mouse LD_{50} U) and has not yet been tested in BoNT/B antibody detection, the assay is likely adaptable to BoNT/B antibody detection.

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